

1/PRTS

09/674330

534 Rec'd PCT/PTO 30 OCT 2000

### Specification

A novel polypeptide, a cDNA encoding the polypeptide and utilization thereof

### Field of the Invention

The present invention provides a novel polypeptide, a cDNA encoding the polypeptide, and utilization thereof.

### Background of the Invention

In modern medical research, cardiovascular biology is a field that attracts considerable attention because cardiovascular disease is the leading cause of mortality. Cardiovascular research has revealed important facts about neointimal formation and arterial remodeling, both of which are thought to contribute to plaque formation in atherosclerosis and blood vessel narrowing. For example, there are three aspects of the cellular process in hypercholesterolaemia induced blood vessel damage in animal models that mimic human development of arteriosclerotic coronary disease. The three elements that form lesions on the artery wall are: a) proliferation of smooth muscle cells, macrophages and lymphocytes, b) formation of connective tissues (mainly elastic fiber proteins, collagen and proteoglycans made by smooth muscle cells in a process similar to scar formation), and c) the accumulation of lipid and cholesterol in the newly formed connective tissue matrices. The exact sequence of the three damaging elements are debatable, but it is clear that the abnormal dedifferentiation, redifferentiation and growth of smooth muscle cells contribute structurally to vessel damage. Moreover, another significant pathological process that involves abnormal smooth muscle cell growth is restenosis after Percutaneous transluminal coronary angioplasty (PTCA).

The present inventors made reasonable efforts, by isolation of the molecules related to participation of smooth muscle cells in angiogenesis, for the aim to utilize them for regulation of abnormal proliferation of smooth muscle cells such like described above.

In order to obtain a certain polypeptide or cDNA coding for the same, there has been generally employed a method composed of detecting the aimed biological activity in a tissue or a cell culture medium, then identifying a polypeptide as substance of the activity through the isolation and purification and isolating a gene encoding the polypeptide or expression-cloning method to isolate a gene by access of the biological activity of the polypeptide encoded by it.

Because in many cases, however, physiologically active polypeptides have various biological activities, when taking the method to approaches based on a certain activity to isolate a gene, it has increasingly been happened that the gene is turned out to be identical to a known gene which has another activity after spending much efforts to isolate it. And because, in many cases, biological factors are produced only in a very slight amount or only in a specific condition, it is often made difficult to isolate and purify a factor and detect its biological activity.

Recent rapid developments in techniques for constructing cDNAs and sequencing techniques have made it possible to quickly sequence a large amount of cDNAs. By utilizing these techniques, a process, which comprises constructing cDNAs at random, identifying the nucleotide sequences thereof, expressing novel polypeptides encoded by them, is now in progress. Although this process is advantageous in that a gene can be cloned and information regarding its nucleotide sequence can be obtained without any biochemical or genetic analysis, the target gene can be discovered thereby only accidentally in many cases.

### Disclosure of the Invention

The present inventors investigated to find novel factors (polypeptides) which are useful for study or for the treatment or diagnosis of diseases induced by abnormal proliferation of smooth muscle. Especially, we had aimed secreted proteins and membrane proteins which have signal sequences for secretion.

The present inventors have studied cloning method of genes coding proliferation and/or differentiation factors functioning in hematopoietic systems and immune systems. Focusing their attention on the fact that most of the secretory proteins such as proliferation and/or differentiation factors (for example various cytokines) and membrane proteins such as receptors thereof (hereafter these proteins will be referred to generally as secretory proteins and the like) have sequences called signal peptides in the N-termini, the inventors conducted extensive studies on a process for efficiently and selectively cloning a gene coding for a signal peptide. Finally, we have successfully invented a screening method for cDNAs having sequence encoding signal peptides, we called the method as signal sequence trap (SST) (Japanese Patent Publication No. 6-315380).

We also developed yeast SST method on the same concept. By the method using yeast, genes including sequence encoding signal peptide can be identified more easily and effectively (USP No. 5,536,637).

By using the present method, the present inventors identified novel secreted protein produced by mouse embryonic heart and a cDNA fragments encoding them, and by using the sequence information of the cDNA fragments they isolated each full-length cDNA from mouse embryonic heart and human kidney. And they discovered that the polypeptides had functions to suppress smooth muscle cells.

The present cDNA sequence was named as a clone mouse A55 and isolated from cDNA library derived from mouse embryonic heart based on genetic information obtained by using the Yeast SST method described above. The clone, mouse A55 is a full-length cDNA encoding a secreted polypeptide (which is called mouse A55 polypeptide here).

There was no DNA sequence which is identical to that of mouse and human A55 of the present invention, when DNA sequence of mouse A55 was compared with data base by BLASTN and FASTA. And there was no polypeptides which is identical to that of mouse and human A55 of the present invention, when amino acid sequence of mouse and human A55 was compared with data base by BLASTX, BLASTP and FASTA. So the polypeptides of the present invention are considered to be novel.

The inventors discovered that the polypeptides had functions to suppress smooth muscle cells. Accordingly, the polypeptides may be useful for treatment of diseases related to abnormal proliferation of smooth muscle cells, for example, arteriosclerotic coronary disease, neointimal formation which results in restenosis after percutaneous transluminal coronary angioplasty and myosarcoma.

The present invention provides:

- 1) a polypeptide comprising an amino acid sequence shown in SEQ ID NO. <sup>3, 4, 8, or 9</sup> ~~1, 4, 6 or 2,~~
- 2) a cDNA encoding the polypeptide described above (1),
- 3) a cDNA having a nucleotide sequence shown in SEQ ID NO. <sup>1, 5, 6</sup> ~~2, 5, 7~~ or 10,
- 4) a cDNA that consists of a nucleotide sequence shown in SEQ ID NO. <sup>2 or 7</sup> ~~3, 8 or 13.~~

#### Brief Description of Figures

Fig. 1 It shows that mouse A55 protein inhibits proliferation of rat aortic vascular smooth muscle cells which ~~was~~ stimulated by PDGF.

### Detailed Discliption

The present invention is concerned with a polypeptide that comprising the amino acid sequence shown in SEQ ID NO. <sup>3, 4, 8, 9</sup>~~1, 4, 6~~ or ~~2~~ in substantially purified form, a homologue thereof, a fragment of the sequence and a homologue of the fragment.

Further, the present invention is concerned with a cDNA encoding the above peptides. More particularly the present invention is provided cDNA comprising the nucleotide sequence shown in SEQ ID NO. <sup>1, 5, 6, or 10</sup>~~2, 5, 7 or 10~~, and cDNA containing a fragment which is selectively hybridizing to the cDNA that comprising nucleotide sequence shown in SEQ ID NO. <sup>1, 5, 6</sup>~~2, 5, 7~~ or 10. Complementary sequence of the above nucleotide sequence is also included in cDNA selectively hybridized. Hybridization are performed in an stringent condition.

A polypeptide comprising amino acid sequence shown in SEQ ID NO. <sup>3</sup>~~1, 4, 6 or 9~~ <sup>4, 8, or 9</sup> in substantially purified form will generally comprise the polypeptide in a preparation in which more than 90%, e.g. 95%, 98% or 99% of the polypeptide in the preparation is that of the SEQ ID NO. <sup>3, 4, 8</sup>~~1, 4, 6~~ or 9.

A homologue of polypeptide comprising amino acid sequence shown in SEQ ID NO. <sup>3, 4, 8, or 9</sup>~~1, 4, 6 or 9~~ will be generally at least 70%, preferably at least 80 or 90% and more preferably at least 95% homologous to the polypeptide of SEQ ID NO. <sup>3</sup>~~1~~ over a region of at least 20, preferably at least 30, for instance 40, 60 or 100 more contiguous amino acids. Such a polypeptide homologue will be referred to a polypeptide of the present invention.

Generally, a fragment of polypeptide comprising amino acid sequence

shown in SEQ ID NO. ~~1, 4, 6 or 9~~ <sup>3, 7, 8, or</sup> or its homologues will be at least 10, preferably at least 15, for example 20, 25, 30, 40, 50 or 60 amino acids in length, and are also referred to by the term "a polypeptide of the present invention".

A cDNA capable of selectively hybridizing to the cDNA comprising a nucleotide sequence shown in SEQ ID NO. <sup>1, 5, 6, or 10</sup> ~~2, 5, 7 or 10~~ will be generally at least 70%, preferably at least 80 or 90% and more preferably at least 95% homologous to the cDNA of SEQ ID NO. <sup>1, 5, 6, or 10</sup> ~~2, 5, 7 or 10~~ over a region of at least 20, preferably at least 30, for instance 40, 60 or 100 or more contiguous nucleotides. Such cDNA will be referred to "a cDNA of the present invention".

Fragments of the cDNA comprising nucleotide sequence shown in SEQ ID NO. <sup>1, 5, 6</sup>~~2, 5, 7~~ or 10 will be at least 10, preferably at least 15, for example 20, 25, 30 or 40 nucleotides in length, and will be also referred to "a cDNA of the present invention" as used herein.

A further embodiment of the present invention provides replication and expression vectors carrying cDNA of the invention. The vectors may be, for example, plasmid, virus or phage vectors provided with an origin of replication, optionally a promoter for the expression of the said cDNA and optionally a regulator of the promoter. The vector may contain one or more selectable marker genes, for example a ampicillin resistance gene. The vector may be used in vitro, for example of the production of RNA corresponding to the cDNA, or used to transfect or transfect a host cell.

A further embodiment of the present invention provides host cells transformed with the vectors for the replication and expression of the cDNA of the invention, including the nucleotide sequence shown in SEQ ID NO. <sup>1</sup>~~1~~, 2, 5, 6, 7, ~~3, 5, 7, 8~~ or 10 or the open reading frame thereof. The cells will be chosen to be compatible with the vector and may for example be bacterial, yeast, insect or mammalian.

A further embodiment of the present invention provides a method of

producing a polypeptide which comprises culturing host cells of the present invention under conditions effective to express a polypeptide of the invention. Preferably, in addition, such a method is carried out under conditions in which the polypeptide of the invention is expressed and then produced from the host cells.

cDNA of the present invention may also be inserted into the vectors described above in an antisense orientation in order to provide for the production of antisense RNA. Such antisense RNA may be used in a method of controlling the levels of a polypeptide of the invention in a cell.

The invention also provides monoclonal or polyclonal antibodies against a polypeptide of the invention. The invention further provides a process for the production of monoclonal or polyclonal antibodies to the polypeptides of the invention. Monoclonal antibodies may be prepared by common hybridoma technology using polypeptides of the invention or fragments thereof, as an immunogen. Polyclonal antibodies may also be prepared by common means which comprise inoculating host animals, for example a rat or a rabbit, with polypeptides of the invention and recovering immune serum.

The present invention also provides pharmaceutical compositions containing a polypeptide of the invention, or an antibody thereof, in association with a pharmaceutically acceptable diluent and/or carrier.

The polypeptide of the present invention includes that which a part of their amino acid sequence is lacking (e.g., a polypeptide comprised of the only essential sequence for revealing a biological activity in an amino acid sequence shown in SEQ ID NO. <sup>3</sup>~~1~~), that which a part of their amino acid sequence is replaced by other amino acids (e.g., those replaced by an amino acid having a similar property) and that which other amino acids are added or inserted into a part of their amino acid sequence, as well as those comprising the amino acid sequence shown in SEQ ID NO. <sup>3,4,8</sup>~~1,4,6~~ or 9.

As known well, there are one to six kinds of codon as that encoding one amino acid (for example, one kind of codon for Methioine (Met), and six kinds of codon for leucine (Leu) are known). Accordingly, the nucleotide sequence of cDNA can be changed in order to encode the polypeptide having the same amino acid sequence.

The DNA of the present invention, specified in (2) includes a group of every nucleotide sequences encoding polypeptides (1) shown in SEQ ID NO. <sup>3,4,8</sup>~~1,4,6~~ or 9. There is a probability that yield of a polypeptide is improved by changing a nucleotide sequence.

The cDNA specified in (3) is the embodiment of the cDNA shown in (2), and indicate the sequence of natural form.

The cDNA shown in (4) indicates the sequence of the cDNA specified in (3) with natural non-translational region.

cDNA carrying nucleotide sequence shown in SEQ ID NO. <sup>2 7</sup>~~1 8~~ or ~~8~~ is prepared by the following method:

Brief description of Yeast SST method (see USP No. 5,536,637) is as follows.

Yeast such as *Saccharomyces cerevisiae* should secrete invertase into the medium in order to take sucrose or raffinose as a source of energy or carbon. (Invertase is an enzyme to cleave raffinose into sucrose and melibiose, sucrose into fructose and glucose.) It is known that many known mammalian signal peptide make yeast secrete its invertase. From these knowledge, SST method was developed as a screening method to find novel signal peptide which make it possible can to secrete yeast invertase from mammalian cDNA library. SST method uses yeast growth on raffinose medium as a marker. Non-secretory type invertase gene SUC2 (GENBANK Accession No. V01311) lacking initiation codon ATG was inserted to yeast expression vector to prepare yeast SST vector pSUC2.

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In this expression vector, ADH promoter, ADH terminator (both were derived from AAH5 plasmid (Gammerer, Methods in Enzymol. 101, 192-201, 1983)), 2u ori (as a yeast replication origin), TRP1 (as a yeast selective marker), ColE1 ori (as a E. Coli replication origin) and ampicillin resistance gene (as a drug resistance marker) were inserted. Mammalian cDNA was inserted into the upstream of SUC2 gene to prepare yeast SST cDNA library. Yeast lacking secretory type invertase, was transformed with this library. If inserted mammalian cDNA encodes a signal peptide, yeast could be survive in raffinose medium as a result of restoring secretion of invertase. Only to culture yeast colonies, prepare plasmids and determine the nucleotide sequence of the insert cDNAs, it is possible to identify novel signal peptide rapidly and easily.

Preparation of yeast SST cDNA library is as follows:

- (1) mRNA is isolated from the targeted cells, second-strand synthesis is performed by using random primer with certain restriction enzyme (enzyme I) recognition site,
- (2) double-strand cDNA is ligated to adapter containing certain restriction endonuclease (enzyme II) recognition site, differ from enzyme I, digested with enzyme I and fractionated in a appropriate size,
- (3) obtained cDNA fragment is inserted into yeast expression vector on the upstream region of invertase gene which signal peptide is deleted and the library was transformed.

Detailed description of each step is as follows:

- (1) mRNA is isolated from mammalian organs and cell lines stimulate them with appropriate stimulator if necessary) by known methods (Molecular Cloning (Sambrook, J., Fritsch, E. F. and Maniatis, T., Cold Spring Harbor Laboratory Press, 1989) or Current Protocol in Molecular Biology (F. M. Ausubel et al, John Wiley & Sons, Inc.) if not remark especially).

Mouse embryonic heart is chosen as a tissue source. Double-strand cDNA synthesis using random primer is performed by known methods.

Any sites may be used as restriction endonuclease recognition site I which is linked to adapter and restriction endonuclease recognition site II which is used in step (2), if both sites are different each other. Preferably, XhoI is used as enzyme I and EcoRI as enzyme II.

In step (2), cDNA is created blunt-ends with T4 DNA polymerase, ligated enzyme II adapter and digested with enzyme I. Fragment cDNA is analyzed with agarose-gel electrophoresis and is selected cDNA fraction ranging in size from 300 to 800 bp. As mentioned above, any enzyme may be used as enzyme II if it is not same the enzyme I.

In step (3), cDNA fragment obtained in step (2) is inserted into yeast expression vector on the upstream region of invertase gene which signal peptide is deleted. E. coli transformed with the expression vector. Many vectors are known as yeast expression plasmid vector. For example, YEp24 is also functioned in E. Coli. Preferably pSUC2 as described above is used.

Many host E. Coli strains are known for transformation, preferably DH10B competent cell is used. Any known transformation method is available, preferably it is performed by electroporation method. Transformant is cultured by known methods to obtain cDNA library for yeast SST method.

However not every All of the clones do not contain cDNA fragment. Further all of the gene fragments do not encode unknown signal peptides. It is therefore necessary to screen a gene fragment encoding for an unknown signal peptide from the library.

Therefore, screening of fragments containing a sequence encoding an appropriate signal peptide is performed by transformation of the cDNA library into *Saccharomyces cerevisiae* (e.g. Y2455 strain) which lack invertase (it may be prepared by known methods.). Transformation of yeast is performed

by known methods, e.g. lithium acetate method. Transformant is cultured in a selective medium, then transferred to a medium containing raffinose as a carbon source. Survival colonies are selected and then prepared plasmid. Survival colonies on a raffinose-medium indicates that some signal peptide of secretory protein was inserted to this clone.

Isolated positive clones is determined the nucleotide sequence. As to a cDNA encodes unknown protein, full-length clone may be isolated by using cDNA fragment as a probe and then determined to obtain full-length nucleotide sequence. These manipulation is performed by known methods.

Once the nucleotide sequences shown in SEQ ID NO. 2, 5, 7 or 10 are determined partially or preferably fully, it is possible to obtain cDNA encode mammalian protein itself, homologue or subset of the invention.

cDNA library or mRNA derived from mammals was screened by PCR with any synthesized oligonucleotide primers or by hybridization with any fragment as a probe. It is possible to obtain cDNA encodes other mammalian homologue protein from other mammalian cDNA or genome library.

If a cDNA obtained above contains a nucleotide sequence of cDNA fragment obtained by SST (or ~~consensus~~ consensus sequence thereof), it will be thought that the cDNA encodes signal peptide. So it is clear that the cDNA will be full-length or almost full. (All signal peptides exist at N-termini of a protein and are encoded at 5'-temini of open reading frame of cDNA.)

The confirmation may be carried out by Northern analysis with the said cDNA as a probe. It is thought that the cDNA is almost complete length, if length of the cDNA is almost the same length of the mRNA obtained in the hybridizing band.

The present invention supplies full-length protein and also its mature protein sequence. The full-length protein sequence deduced from nucleotide sequences shown in SEQ ID NO. <sup>1</sup><sub>2</sub> or <sup>6</sup><sub>7</sub>. Mature proteins are obtained by

expressing full-length cDNAs shown in SEQ ID NO. <sup>2 7</sup><sub>1, 3</sub> or <sup>2 7</sup><sub>8</sub> in mammalian cells or other host cells. Mature protein sequences are deduced from their full-length amino acid sequences.

Once the nucleotide sequences shown in SEQ ID NOS <sup>1, 5, 6</sup><sub>1, 2, 5, 7</sub> or 10 are determined, cDNAs of the present invention are obtained by chemical synthesis, or by hybridization making use of nucleotide fragments which are chemically synthesized as a probe. Furthermore, cDNAs of the present invention are obtained in desired amount by transforming a vector that contains the cDNA into a proper host, and culturing the transformant.

The polypeptides of the present invention may be prepared by:

- (1) isolating and purifying from an organism or a cultured cell,
  - (2) chemically synthesizing, or
  - (3) using recombinant DNA technology,
- preferably, by the method described in (3) in industrial production.

Examples of expression system for (host-vector system) producing a polypeptide by using recombinant DNA technology are the expression systems of bacteria, yeast, insect cells and mammalian cells.

In the expression of the polypeptide, for example, in E. Coli, the expression vector is prepared by adding the initiation codon (ATG) to 5' end of a DNA encoding mature peptide, connecting the DNA thus obtained to the downstream of a proper promoter (e.g., trp promoter, lac promoter,  $\lambda$  PL promoter, T7 promoter etc.), and then inserting it into a vector (e.g., pBR322, pUC18, pUC19 etc.) which functions in an E. coli strain.

Then, an E. coli strain (e.g., E. coli DH1 strain, E. coli JM109 strain, E. coli HB101 strain, etc.) which is transformed with the expression vector described above may be cultured in a appropriate medium to obtain the desired polypeptide. When a signal peptide of bacteria (e.g., signal peptide of pel B) is utilized, the desired polypeptide may be also released in periplasm.

Furthermore, a fusion protein with other polypeptide may be also produced easily.

In the expression of the polypeptide, for example, in a mammalian cells, for example, the expression vector is prepared by inserting the DNA shown in SEQ ID NO. <sup>1, 5, 4</sup>~~2, 5, 7~~ or 10 into the downstream of a proper promoter (e.g., SV40 promoter, LTR promoter, metallothionein promoter etc.) in a proper vector (e.g., retrovirus vector, papilloma virus vector, vaccinia virus vector, SV40 vector, etc.) a proper mammalian cell (e.g., monkey COS-7 cell, Chinese hamster CHO cell, mouse L cell etc.) is transformed with the expression vector thus obtained, and then the transformant is cultured in a proper medium to get a desired polypeptide in the culture medium. Further, fusion protein may be produced by linking cDNA fragment encoding other polypeptide such as Fc portion of an antibody. The polypeptide thus obtained may be isolated and purified by conventional biochemical methods.

### Industrial Utility

The polypeptides of the present invention and cDNA encoding them are expected to exhibit one or more of the uses or biological activities (including those associated with assays cited herein) identified below.

Uses or activities described for proteins of the present invention may be provided by administration or use of such proteins or by administration or use of cDNA encoding them (such as, for example, in gene therapies or vectors suitable for introduction of DNA).

We have been confirmed that the said polypeptide possess the suppressing activity on the differentiation of vascular smooth muscle cells. Accordingly, the polypeptides may be useful for treatment of diseases related to abnormal proliferation of smooth muscle cells, for example, arteriosclerotic coronary disease, neointimal formation which results in

restenosis after percutaneous transluminal coronary angioplasty and myosarcoma.

But not limit the present invention, the present polypeptide may show the following activity :

<Cytokine activity and cell proliferation/differentiation activity>

The protein of the present invention may exhibit cytokine, cell proliferation (either inducing or inhibiting) or cell differentiation (either inducing or inhibiting) activity or may induce production of other cytokines in certain cell populations.

Many protein factors discovered to date, including all known cytokines, have exhibited activity in one or more factor dependent cell proliferation assays, and hence the assays serve as a convenient confirmation of cytokine activity.

The activity of a protein of the present invention is evidenced by any one of a number of routine factor dependent cell proliferation assays for cell lines.

<Immune stimulating/suppressing activity>

The protein of the present invention may also exhibit immune stimulating or immune suppressing activity. The protein may be useful in the treatment of various immune deficiencies and disorders (including severe combined immunodeficiency (SCID)), e.g., in regulating (up or down) growth and proliferation of T and/or B lymphocytes, as well as effecting the cytolytic activity of NK cells and other cell populations.

These immune deficiencies may be genetic or be caused by viral (e.g. HIV) as well as bacterial or fungal infections, or may result from autoimmune disorders.

More specifically, infectious diseases caused by viral, bacterial, fungal or other infection may be treatable using the protein of the present

invention, including infections by HIV, hepatitis viruses, herpes viruses, mycobacteria, leishmania, malaria and various fungal infections such as candida. Of course, in this regard, a protein of the present invention may also be useful where a boost to the immune system generally would be indicated, i.e., in the treatment of cancer.

Such a protein of the present invention may also be useful in the treatment of allergic reactions and conditions, such as asthma or other respiratory problems.

The protein of the present invention may also suppress chronic or acute inflammation, such as, for example, that associated with infection (such as septic shock or systemic inflammatory response syndrome (SIRS)), inflammatory bowel disease, Crohn's disease or resulting from overproduction of cytokines such as TNF or IL-1 (such as the effect demonstrated by IL-11).

<Hematopoiesis regulating activity>

The protein of the present invention may be useful in regulation of hematopoiesis and, consequently, in the treatment of myeloid or lymphoid cell deficiencies. Even marginal biological activity in support of colony forming cells or of factor-dependent cell lines indicates involvement in regulating hematopoiesis.

The said biological activities are concerned with the following all or some example(s). e.g. in supporting the growth and proliferation of erythroid progenitor cells alone or in combination with other cytokines, thereby indicating utility.

for example, in treating various anemias or for use in conjunction with irradiation/chemotherapy to stimulate the production of erythroid precursors and/or erythroid cells;

in supporting the growth and proliferation of myeloid cells such as

granulocytes and monocytes/macrophages (i.e., traditional CSF activity) useful, for example, in conjunction with chemotherapy to prevent or treat consequent myelo-suppression;

in supporting the growth and proliferation of megakaryocytes and consequently of platelets thereby allowing prevention or treatment of various platelet disorders such as thrombocytopenia, and generally for use in place of or complimentary to platelet transfusions;

and/or in supporting the growth and proliferation of hematopoietic stem cells which are capable of maturing to any and all of the above-mentioned hematopoietic cells and therefore find therapeutic utility in various stem cell disorders (such as those usually treated with transplantation, including, without limitation, aplastic anemia and paroxysmal nocturnal hemoglobinuria), as well as in repopulating the stem cell compartment post irradiation/chemotherapy, either in-vivo or ex-vivo (i.e. in conjunction with bone marrow transplantation) as normal cells or genetically manipulated for gene therapy.

Suitable assays for proliferation and differentiation of various hematopoietic lines are cited above.

The activity of a protein of the invention may, among other means, be measured by the following methods :

<Tissue generation/regeneration activity>

The protein of the present invention also may have utility in compositions used for bone, cartilage, tendon, Ligament and/or nerve tissue growth or regeneration, as well as for wound healing and tissue repair, and in the treatment of burns, incisions and ulcers.

The protein of the present invention, which induces cartilage and/or bone growth in circumstances where bone is not normally formed, has application in the healing of bone fractures and cartilage damage or defects

in humans and other animals. Such a preparation employing the protein of the invention may have prophylactic use in closed as well as open fracture reduction and also in the improved fixation of artificial joints. De novo bone formation induced by an osteogenic agent contributes to the repair of congenital, trauma induced, or oncologic resection induced craniofacial defects, and also is useful in cosmetic plastic surgery.

The protein of this invention may also be used in the treatment of periodontal disease, and in other tooth repair processes. Such agents may provide an environment to attract bone-forming cells, stimulate growth of bone-forming cells or induce differentiation of progenitors of bone-forming cells. The protein of the invention may also be useful in the treatment of osteoporosis or osteoarthritis, such as through stimulation of bone and/or cartilage repair or by blocking inflammation or processes of tissue destruction (collagenase activity, osteoclast activity, etc.) mediated by inflammatory processes.

Another category of tissue regeneration activity that may be attributable to the protein of the present invention is tendon/ligament formation. A protein of the present invention, which induces tendon/ligament-like tissue or other tissue formation in circumstances where such tissue is not normally formed, has application in the healing of tendon or ligament tears, deformities and other tendon or ligament defects in humans and other animals. Such a preparation employing a tendon/Ligament-like tissue inducing protein may have prophylactic use in preventing damage to tendon or ligament tissue, as well as use in the improved fixation of tendon or ligament to bone or other tissues, and in repairing defects to tendon or ligament tissue. De novo tendon/ligament-like tissue formation induced by a composition of the present invention contributes to the repair of congenital, trauma induced, or other tendon or ligament defects of other

origin, and is also useful in cosmetic plastic surgery for attachment or repair of tendons or ligaments.

The compositions of the present invention may provide an environment to attract tendon- or ligament-forming cells, stimulate growth of tendon- or ligament-forming cells, induce differentiation of progenitors of tendon- or ligament-forming cells, or induce growth of tendon Ligament cells or progenitors ex vivo for return in vivo to effect tissue repair.

The compositions of the invention may also be useful in the treatment of tendinitis, carpal tunnel syndrome and other tendon or ligament defects. The compositions may also include an appropriate matrix and/or sequestering agent as a carrier as is well known in the art.

The protein of the present invention may also be useful for proliferation of neural cells and for regeneration of nerve and brain tissue. i.e. for the treatment of central and peripheral nervous system diseases and neuropathies. as well as mechanical and traumatic disorders, which involve degeneration, death or trauma to neural cells or nerve tissue.

More specifically, a protein may be used in the treatment of diseases of the peripheral nervous system, such as peripheral nerve injuries, peripheral neuropathy and localized neuropathies, and central nervous system diseases, such as Alzheimer's, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and Shy-Drager syndrome.

Further conditions which may be treated in accordance with the present invention include mechanical and traumatic disorders, such as spinal cord disorders, head trauma and cerebrovascular diseases such as stroke. Peripheral neuropathies resulting from chemotherapy or other medical therapies may also be treatable using a protein of the invention.

It is expected that the protein of the present invention may also exhibit activity for generation of other tissues, such as organs (including,

for example, pancreas, liver, intestine, kidney, skin, endothelium), muscle (smooth, skeletal or cardiac) and vascular (including vascular endothelium) tissue, or for promoting or suppressing the proliferation of cells comprising such tissues. Part of the desired effects may be by inhibition of fibrotic scarring to allow normal tissue to regenerate.

A protein of the present invention may also be useful for gut protection or regeneration and treatment of lung or liver fibrosis, reperfusion injury in various tissues, and conditions resulting from systemic cytokine damage.

<Activin/Inhibin activity>

The protein of the present invention may also exhibit activin- or inhibin-related activities. Inhibins are characterized by their ability to inhibit the release of follicle stimulating hormone (FSH), while activins and are characterized by their ability to stimulate the release of follicle stimulating hormone (FSH).

Thus, a protein of the present invention, alone or in heterodimers with a member of the inhibin \*a family, may be useful as a contraceptive based on the ability of inhibins to decrease fertility in female mammals and decrease spermatogenesis in male mammals. Administration of sufficient amounts of other inhibins can induce infertility in these mammals.

Alternatively, the protein of the invention, as a homodimer or as a heterodimer with other protein subunits of the inhibin-\*b group, may be useful as a fertility inducing therapeutic, based upon the ability of activin molecules in stimulating FSH release from cells of the anterior pituitary. See for example, USP 4,798,885. The polypeptide of the invention may also be useful for advancement of the onset of fertility in sexually immature mammals, so as to increase the lifetime reproductive performance of domestic animals such as cows, sheep and pigs.

<Chemotactic/chemokinetic activity>

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A protein of the present invention may have chemotactic or chemokinetic activity (e.g., act as a chemokine) for mammalian cells, including. for example. monocytes, neutrophils, T-cells, mast cells, eosinophils and/or endothelial cells.

Chemotactic and chemokinetic proteins can be used to mobilized or attract a desired cell 'population to a desired site of action. Chemotactic or chemokinetic proteins provide particular advantages in treatment of wounds and other trauma to tissues, as well as in treatment of localized infections. For example, attraction of lymphocytes, monocytes or neutrophils to tumors or sites of infection may result in improved immune responses against the tumor or infecting agent.

A protein or peptide has chemotactic activity for a particular cell population if it can stimulate, directly or indirectly, the directed orientation or movement of such cell population. Preferably, the protein or peptide has the ability to directly stimulate directed movement of cells. Whether a particular protein has chemotactic activity for a population of cells can be readily determined by employing such protein or peptide in any known assay for cell chemotaxis.

#### <Hemostatic and thrombolytic activity>

The protein of the invention may also exhibit hemostatic or thrombolytic activity. As a result, such a protein is expected to be useful in treatment of various coagulation disorders (including hereditary disorders, such as hemophiliias) or to enhance coagulation and other hemostatic events in treating wounds resulting from trauma, surgery or other causes. A protein of the invention may also be useful for dissolving or inhibiting formation of thromboses and for treatment and prevention of conditions resulting therefrom (such as. for example, infarction or stroke).

#### <Receptor/ligand activity>



adhesion properties linked to tumor growth and metastasis. Cadherin malfunction is also implicated in other human diseases, such as pemphigus vulgaris and pemphigus foliaceus (autoimmune blistering skin diseases), Crohn's disease, and some developmental abnormalities.

The cadherin superfamily includes well over forty members, each with a distinct pattern of expression. All members of the superfamily have in common conserved extracellular repeats (cadherin domains), but structural differences are found in other parts of the molecule. The cadherin domains bind calcium to form their tertiary structure and thus calcium is required to mediate their adhesion. Only a few amino acids in the first cadherin domain provide the basis for homophilic adhesion; modification of this recognition site can change the specificity of a cadherin so that instead of recognizing only itself, the mutant molecule can now also bind to a different cadherin. In addition, some cadherins engage in heterophilic adhesion with other cadherin.

E-cadherin, one member of the cadherin superfamily, is expressed in epithelial cell types. Pathologically, if E-cadherin expression is lost in a tumor, the malignant cells become invasive and the cancer metastasizes. Transfection of cancer cell line with cDNAs expressing E-cadherin has reversed cancer-associated changes by returning altered cell shapes to normal, restoring cells adhesiveness to each other and to their substrate, decreasing the cell growth rate, and drastically reducing anchorage-independent cell growth.

Thus, reintroducing E-cadherin expression reverts carcinomas to a less advanced stage. It is likely that other cadherins have the same invasion suppresser role in carcinomas derived from other tissue types. Therefore, proteins of the present invention with cadherin activity, and cDNAs of the present invention encoding such proteins, can be used to treat cancer.

Introducing such proteins or cDNAs into cancer cells can reduce or eliminate the cancerous change observed in these cells by providing normal cadherin expression.

Cancer cells have also been shown to express cadherins of a different tissue type than their origin, thus allowing these cells to invade and metastasize in a different tissue in the body. Proteins of the present invention with cadherin activity, and cDNAs of the present invention encoding such proteins, can be substituted in these cells for the inappropriately expressed cadherins, restoring normal cell adhesive properties and reducing or eliminating the tendency of the cells to metastasize.

Additionally, proteins of the present invention with cadherin activity, and cDNA of the present invention encoding such proteins, can be used to generate antibodies recognizing and binding to cadherins. Such antibodies can be used to block the adhesion of inappropriately expressed tumor-cell cadherins, preventing the cells from forming a tumor elsewhere. Such an anti-cadherin antibody can also be used as a marker for the grade, pathological type, and prognosis of a cancer, i.e. the more progressed the cancer, the less cadherin expression there will be, and this decrease in cadherin expression can be detected by the use of a cadherin-binding antibody.

Fragments of proteins of the present invention with cadherin activity, preferably a polypeptide comprising a decapeptide of the cadherin recognition site, and cDNAs of the present invention encoding such protein fragments, can also be used to block cadherin function by binding to cadherins and preventing them from binding in ways that produce undesirable effects.

Additionally, fragments of proteins of the present invention with cadherin activity, preferably truncated soluble cadherin fragments which have been found to be stable in the circulation of cancer patients, and polynucleotides encoding such protein fragments, can be used to disturb

proper cell-cell adhesion.

<Tumor Inhibiting activity>

In addition to the activities described above for immunological treatment or prevention of tumors, the protein of the invention may exhibit other anti-tumor activities. The protein may inhibit tumor growth directly or indirectly (such as, for example, via ADCC). The protein may exhibit its tumor inhibitory activity by acting on tumor tissue or tumor precursor tissue, by inhibiting formation of tissues necessary to support tumor growth (such as, for example, by inhibiting angiogenesis), by causing production of other factors, agents or cell types which inhibit tumor growth, or by suppressing, eliminating or inhibiting factors, agents or cell types which promote tumor growth.

<Other activity>

The protein of the invention may also exhibit one or more of the following additional activities or effects: inhibiting the growth, infection or function of, or killing, infectious agents, including, bacteria, viruses, fungi and other parasites;

effecting (suppressing or enhancing) bodily characteristics, including, height, weight, hair color, eye color, skin, fat to lean ratio or other tissue pigmentation, or organ or body part size or shape (such as, for example, breast augmentation or diminution);

effecting elimination of dietary fat, protein, carbohydrate;

effecting behavioral characteristics, including appetite, libido, stress, cognition (including cognitive disorders), depression and violent behaviors;

providing analgesic effects or other pain reducing effects;

promoting differentiation and growth of embryonic stem cells in lineages other than hematopoietic lineages;

in the case of enzymes, correcting deficiencies of the enzyme and treating deficiency-related diseases.

The polypeptide with above activities, is suspected to have following functions by itself or interaction with its ligands or receptors or association with other molecules. For example, proliferation or cell death of B cells, T cells and/or mast cells or class specific induction of B cells by promotion of class switch of immunoglobulin genes; differentiation of B cells to antibody-forming cells; proliferation, differentiation, or cell death of precursors of granulocytes; proliferation, differentiation, or cell death of precursors of monocytes-macrophages; proliferation, of up regulation or cell death of neutrophils, monocytes-macrophages, eosinophils and/or basophils; proliferation, or cell death of precursors of megakaryocytes; proliferation, differentiation, or cell death of precursors of neutrophils; proliferation, differentiation, or cell death of precursors of T cells and B cells; promotion of production of erythrocytes; sustainment of proliferation of erythrocytes, neutrophils, eosinophils, basophils, monocytes-macrophages, mast cells, precursors of megakaryocyte ; promotion of migration of neutrophils, monocytes-macrophages, B cells and/or T cells; proliferation or cell death of thymocytes; suppression of differentiation of adipocytes; proliferation or cell death of natural killer cells; proliferation or cell death of hematopoietic stem cells; suppression of proliferation of stem cells and each hematopoietic precursor cells; promotion of differentiation from mesenchymal stem cells to osteoblasts or chondrocytes, proliferation or cell death of mesenchymal stem cells, osteoblasts or chondrocytes and promotion of bone absorption by activation of osteoclasts and promotion of differentiation from monocytes to osteoclasts.

This peptide is also suspected to function to nervous system, so

expected to have functions below; differentiation to kinds of neurotransmitter-responsive neurons, survival or cell death of these cells; promotion of proliferation or cell death of glial cells; spread of neural dendrites; survival or cell death of gangriocytes; proliferation, promotion of differentiation, or cell death of astrocytes; proliferation or survival of peripheral neurons; proliferation or cell death of Schwann cells; proliferation, survival or cell death of motoneurons.

Furthermore, in the process of development of early embryonic, this polypeptide is expected to promote or inhibit the organogenesis of epidermis, brain, backbone, and nervous system by induction of ectoderm, that of notochord connective tissues(bone, muscle, tendon), hemocytes, heart, kidney, and genital organs by induction of mesoderm, and that of digestive apparatus (stomach, intestine, liver, pancreas), respiratory apparatus (lung, trachea) by induction of endoderm. In adult, also, this polypeptide is thought to proliferate or inhibit the above organs.

Therefore, this polypeptide itself is expected to be used as an agent for the prevention or treatment of disease of progression or suppression of immune, nervous, or bone metabolic function, hypoplasia or overgrowth of hematopoietic cells: inflammatory disease (rheumatism, ulcerative colitis, etc.), decrease of hematopoietic stem cells after bone marrow transplantation, decrease of leukocytes, platelets, B-cells, or T-cells after radiation exposure or chemotherapeutic dosage against cancer or leukemia, anemia, infectious disease, cancer, leukemia, AIDS, bone metabolic disease(osteoporosis etc.), arteriosclerosis, various degenerative disease (Alzheimer's disease, multiple sclerosis, etc.), or nervous lesion.

In addition, since this polypeptide is thought to induce the differentiation or growth of organs derived from ectoderm, mesoderm, and endoderm, this polypeptide is expected to be an agent for tissue repair

(epidermis, bone, muscle, tendon, heart, kidney, stomach, intestine, liver, pancreas, lung, and trachea, etc.).

Quantitation of this polypeptide in the body can be performed using polyclonal or monoclonal antibodies against this polypeptide. It can be used the study of relationship between this polypeptide and disease or diagnosis of disease, and so on. Polyclonal and monoclonal antibodies can be prepared using this polypeptide or its fragment as an antigen by known method.

Identification, purification or molecular cloning of known or unknown proteins which bind this polypeptide can be performed using this polypeptide by, for example, preparation of the affinity-column.

Identification of the molecules which interact with this polypeptide and molecular cloning of the gene can be performed by west-western method using this polypeptide or by yeast two-hybrid system using the cDNA (preferably cDNA encoding this polypeptide).

Agonists/antagonists of this receptor polypeptide and inhibitors between receptor and signal transduction molecules can be screened using this polypeptide.

For example, the screening can be carried out the following method.

a) The reaction mixtures, which contain this polypeptide, screening compound and the cells, are incubated under the condition which the cells are normally stimulated by this peptide. (The reaction mixtures also contain the labeled compound, which is introduced into the cells according to the cell proliferation, and peptide which allow to observe the function of this peptide efficiently.)

b) Decision that the compounds are efficient agonists/antagonists or not, are performed by measurement of cell proliferation ability.

More detailed methods are followed:

Rat vascular muscle cell line (ATCC CRL-1444 or CRL1476) is cultured

in 96 well plate with 10%FBS for 24 hours. Then the culture medium are replaced to the serum-free medium supplemented with each several concentrations of human PDGF-BB. At that time compounds to screen as well as A55 protein are added in the medium when screening the antagonists of A55 protein. While, compounds alone are added in the medium when screening the agonists of A55 protein. After 24 hours incubation, these cells are pulsed for 4hours with 3H-thymidine. By measuring the 3H-thymidine incorporation, it is possible to determine whether the compounds have inhibitory or stimulatory effect on the A55 activity.

cdNAs of the present invention are useful not only the important and essential template for the production of the polypeptide of the present invention which is expected to be largely useful, but also be useful for diagnosis or therapy (for example, treatment of gene lacking, treatment to stop the expression of the polypeptide by antisense DNA (RNA)).

Genomic DNA may be isolated with the cdNA of the present invention, as a probe. As the same manner, a mouse or human gene encoding which can be highly homologous to the cdNA of the present invention, that is, which encodes a polypeptide highly homologous to the polypeptide of the present invention and a gene of animals excluding mouse or human which can be highly homologous to the cdNA of the present invention, also may be isolated.

#### **Application for Pharmaceuticals**

For the medical treatment for diseases described above, the polypeptide of the invention or the antibody of the polypeptide of the invention may be administered systemically or partially in most cases, usually by oral or parenteral administration, preferably orally, intravenously or intraventricularly.

The doses to be administered depend upon age, body weight, symptom,

desired therapeutic effect, route of administration, and duration of the treatment etc. In human adults, one dose per person is generally between 100  $\mu$ g and 100 mg, by oral administration, up to several times per day, and between 10  $\mu$ g and 100 mg, by parenteral administration up to several times per day.

As mentioned above, the doses to be used depend upon various conditions. Therefore, there are cases in which doses lower than or greater than the ranges specified above may be used.

The compounds of the present invention, may be administered as solid compositions, liquid compositions or other compositions for oral administration, as injections, liniments or suppositories etc. for parenteral administration.

Solid compositions for oral administration include compressed tablets, pills, capsules, dispersible powders, granules. Capsules include soft or hard capsules.

In such compositions, one or more of the active compound(s) is or are admixed with at least one inert diluent (such as lactose, mannitol, glucose, hydroxypropyl cellulose, microcrystalline cellulose, starch, polyvinylpyrrolidone, magnesium metasilicate aluminate, etc.). The compositions may also comprise, as is normal practice, additional substances other than inert diluents: e.g. lubricating agents (such as magnesium stearate etc.), disintegrating agents (such as cellulose calcium glycolate, etc.), stabilizing agents (such as human serum albumin, lactose etc.), and assisting agents for dissolving (such as arginine, asparaginic acid etc.).

The tablets or pills may, if desired, be coated with a film of gastric or enteric materials (such as sugar, gelatin, hydroxypropyl cellulose or hydroxypropylmethyl cellulose phthalate, etc.), or be coated with more than two films. And then, coating may include containment within capsules of

absorbable materials such as gelatin.

Liquid compositions for oral administration include pharmaceutically-acceptable emulsions, solutions, syrups and elixirs. In such compositions, one or more of the active compound(s) is or are contained in inert diluent(s) commonly used (purified water, ethanol etc.). Besides inert diluents, such compositions may also comprise adjuvants (such as wetting agents, suspending agents, etc.), sweetening agents, flavoring agents, perfuming agents, and preserving agents.

Other compositions for oral administration include spray compositions which may be prepared by known methods and which comprise one or more of the active compound(s). Spray compositions may comprise additional substances other than inert diluents: e.g. stabilizing agents (sodium sulfite etc.), isotonic buffer (sodium chloride, sodium citrate, citric acid, etc.). For preparation of such spray compositions, for example, the method described in the United States Patent No. 2,868,691 or 3,095,355 (herein incorporated in their entireties by reference) may be used.

Injectations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions and emulsions. In such compositions, one or more active compound(s) is or are admixed with at least one inert aqueous diluent(s) (distilled water for injection, physiological salt solution, etc.) or inert non-aqueous diluents(s) (propylene glycol, polyethylene glycol, olive oil, ethanol, POLYSOLBATE 80 TM, etc.).

Injectations may comprise additional compound other than inert diluents: e.g. preserving agents, wetting agents, emulsifying agents, dispersing agents, stabilizing agent (such as human serum albumin, lactose, etc.), and assisting agents such as assisting agents for dissolving (arginine, asparaginic acid, etc.).

### The Best Mode of the Invention

The following examples concerning clone A55 are illustrated, but not limit the present invention.

#### Example 1

##### Preparation of poly(A)+RNA

Total RNA was prepared from mouse day18.5 embryonic heart by TRIzol reagent (Trade Mark, GIBCOBRL), and poly (A)<sup>+</sup> RNA was purified from the total RNA by mRNA Purification Kit (Trade Mark, Pharmacia).

#### Example 2

##### Preparation of yeast SST cDNA library

Double strand cDNA was synthesized by SuperScript Plasmid System for cDNA Synthesis and Plasmid Cloning (brand name, GIBCOBRL) with above poly(A)+RNA as template and random 9mer as primer which was containing XhoI site:

a 5'-CGA TTG AAT TCT AGA CCT GCC TCG AGN NNN NNN NN-3' (SEQ ID NO. 11)

cDNA was ligated EcoRI adapter by DNA ligation kit ver.2 (trade name, Takara Shuzo; this kit was used in all ligating steps hereafter.) and digested by XhoI. cDNAs were separated by agarose-gel electrophoresis. 300 - 800 bp cDNAs were isolated and were ligated to EcoRI/NotI site of pSUC2 (see US 5,536,637). E. Coli DH10B strain were transformed by pSUC2 with electroporation to obtain yeast SST cDNA library.

#### Example 3

##### Screening by SST method and DNA sequencing of positive clone

Plasmids of the cDNA library were prepared. Yeast YTK12 strain were

transformed by the plasmids with lithium acetate method (Current Protocols In Molecular Biology 13.7.1). The transformed yeast were plated on triptphan-free medium (CMD-Try medium) for selection. The plate was incubated for 48 hour at 30 oC. Replica of the colony which is obtained by Accutran Replica Plater (trade name, Schleicher & Schuell) were place YPR plate containing raffinose for carbon source, and the plate was incubated for 14 days at 30 oC.

After 3 days, each colony appeared was streaked on YPR plate again. The plates were incubated for 48 hours at 30 oC. Single colony was inoculated to YPR medium and was incubated for 48 hours at 30 oC. Then plasmids were prepared. Insert cDNA was amplified by PCR with two kind primers which exist end side of cloning site on pSUC2 (sense strand primers were biotinylated). Biotinylated single strand of cDNAs were purified with Dynabeads (trade name, DYNAL) and determined the nucleotide sequences.

Sequencing was performed by Dye Terminator Cycle Sequencing Ready Reaction with DNA Sequencing kit (trade name, Applied Biosystems Inc.) and sequence was determined by DNA sequencer 373 (Applied Biosystems Inc.). All sequencing hereafter was carried with this method.

The clone named A55 is not registered on databases by homology search of cDNA sequence and deduced amino acid sequence and so it is cleared that the sequence is novel one. So, we tried to isolate clone full-length cDNA of the fragment of A55 clone (hereafter A55 SST fragment cDNA). We confirmed that A55 SST fragment cDNA contains signal peptide by comparison with known peptide which has signal peptide in view of function and structure.

#### Example 4

#### Cloning and sequencing of a full-length cDNA of A55

Phage particles of a cDNA library of mouse day13 embryonic

heart(uni-ZAP XR, Stratagene) were transfected to E. coli XL1-Blue MRF\* host cells (Stratagene). Obtained one million plaques were transferred to nylon membranes. The membranes were hybridized with 32P-labeled mouse A55 SST fragment cDNA as a probe. Many positive plaques were obtained.

From one positive plaque the phage particles containing a cloned insert were prepared, and were subjected to conversion into phagemid particles (pBluescript SK(-)) by co-infection of E. coli XL1-Blue MRF\* host cells(Stratagene) with ExAssist helper phage(Stratagene). The phagemid particles were transfected to E. coli DH5a. The plasmids were prepared from the obtained transformants.

Nucleotide sequence of 5'-end of cDNA were determined to confirm the existence of the sequences of SST fragment cDNA. And then full-length sequencing were performed to obtain SEQ ID NO. <sup>2</sup>1.

An open reading frame was determined and translation region for amino acid sequence shown in SEQ ID NO. <sup>1</sup>1 and deduced full-length amino acid sequence shown in SEQ ID NO. <sup>3</sup>2 were obtained. Mature protein of the said polypeptide was deduced to 425 amino acids shown in SEQ ID NO. <sup>2</sup>2 (144..1418) or 423 amino acids shown in SEQ ID NO. 4. Translation region of SEQ ID NO. 4 is shown in SEQ ID NO. 5.

It was confirmed that there was no identical sequences to the DNA of the present invention by homology search program, BLASTN and FASTA against public nucleotide database. And it was also confirmed that there were no identical sequences to the polypeptide of the present invention (mouse A55 protein) by homologue search program, BLASTX, BLASTP and FASTA against amino acid database.

It is revealed that the polypeptide of the present invention, mouse A55 is novel secretion protein since the polypeptide have no trans-membrane region by hydrophobisity analysis of the amino acid sequence.

It was revealed that A55 protein contained six EGF like domains by motif search, so it was expected that clone A55 also possesses EGF family like activities. Significant homology were also recognized between the amino acid sequence of clone mouse A55 (1-448 AA region) and the one of human S1-5 (SwissProt Accession No. HSU03877) (1-387 AA region) by the comparison using BLASTX, BLASTP and FASTA. It was reported that human S1-5 was a secreted protein containing EGF like domain, was induced in fibroblasts by growth arrest, and stimulated DNA synthesis (Beata Lecka-Czernik et. al. Mol. Cell. Biol. 15, 120-128, 1995). Farther it was revealed that A55 protein was homologous to many proteins containing EGF-like domain.

#### Example 5

##### Isolation of isoform gene of mouse A55 protein

Initiation codon was determined by cloning of 5'-end cDNA by 5'-RACE (Rapid Amplification of cDNA Ends method using Marathon cDNA Amplification Kit (trade name, Clontech). Double stranded cDNA template was prepared from poly(A)+RNA of mouse embryonic heart tissue. Primer mA55-R1:

5'- CGT TTG TGC ACT GCT GCT GTG CAT TCC -3' (SEQ ID NO. <sup>12</sup><sub>17</sub>)

was prepared based on the information of full-length nucleotide sequences. PCR was performed with the said primer and adapter primer attached in the kit.

Amplified cDNA was separated with agarose-gel electrophoresis, and to pGEM-T Vector (trade name, Promega), ligated in and transformed to E. Coli DH5a and then plasmid was prepared. The full-length nucleotide sequences were determined. We found two deferent 5'-end sequences. One was identical to the clone containing the sequence in SEQ ID NO. <sup>2</sup><sub>17</sub>, the other

contained unknown sequence and no translational start site ATG (See SEQ ID NO. 7 and 8).

The region defined from exon 1 of the clone was replaced by another exon which exists 400 bp downstream region of exon 1 was clarified by gene analysis. So it was cleared that the clone shown in SEQ ID NO. <sup>7</sup>~~8~~ was generated by alternative splicing of exon 1. The clone encodes isoform protein shown in SEQ ID NO. <sup>8</sup>~~7~~ (6 amino acids in N termini of SEQ ID NO. <sup>3</sup>~~2~~ was replaced by 19 amino acids in N termini of SEQ ID NO. <sup>8</sup>~~7~~).

The mature protein of this polypeptide was deduced 425 amino acids shown in SEQ ID NO. <sup>7</sup>~~8~~ (340...1614) or 423 amino acids shown in SEQ ID NO. 9. SEQ ID NO. 10 is the translational region of the polypeptide shown in SEQ ID NO. 9.

#### Example 6

##### Mouse A55 protein expression in mammalian cell

Mouse full-length cDNA shown in SEQ ID NO. <sup>2</sup>~~1~~ was inserted into expression vector for mammalian cell pNotS (Kaufman et al., Nucleic Acids Res. 19, 4485-4490 (1991)) and mouse A55 expression plasmid pNotS-mA55 was constructed.

293T cells (which is derived from 293 cells (ATCC CRL-1573) and it stably transfected with SV40 T antigen) were transfected with pNotS and pNotS-mA55 using lipofection (GIBCOBRL). After preincubated for 19 hours, the cells were pulsed for 30 minutes with <sup>35</sup>S-Met in the Met-free medium. Then the cells were incubated in the medium containing Met for 5 hours. Supernatant of the cells was recovered and concentrated 10-fold using centricon-10 (trade name, AMICON). Samples were subjected to SDS-polyacrylamide-gel electrophoresis. The gel was dried and <sup>35</sup>S-labeled proteins were detected with BAS 2000 (Fuji Film).

A band was detected at 60-70 kDa in the supernatant of pNotS-mA55-transfected 293T cells. This band was not detected in the supernatant of pNotS-transfected 293T cells. This results confirmed that recombinant mouse A55 protein was expressed and secreted into the medium. Molecular weight (60-70 kDa) of this recombinant mouse A55 protein was greater than it (48 kDa) predicted from its amino acid sequences. As this protein had two potential N-linked glycosylation sites and many Ser and Thr residues in which O-linked glycosyl chain could be added, it was suggested that the mouse A55 protein was a glycoprotein.

#### Example 7

Measurement of inhibition on proliferation of rat vascular smooth muscle cells by mouse A55 protein

Vascular smooth muscle cells were isolated from rat aorta ranging from heart to diaphragm and cultured primarily by the methods described in Shin Seikagaku Jikken Kouza 10 (The Japanese Biochemical Society). These cells were co-incubated with 1, 3 or 10 ng/ml of human recombinant PDGF-BB (Genzyme) and 10% (v/v) of the mock or mA55 supernatant prepared according to the method described in example 7. And BrdU incorporation was measured using a Cell Proliferation ELISA, BrdU colorimetric kit (Boehringer-Mannheim).

The supernatant from 293T cells transfected with pNotS-mA55 significantly inhibited BrdU incorporation of rat primary vascular smooth muscle cells, while the supernatant from 293T cells transfected with only pNotS show no effect as shown in Fig. 1.

Moreover the supernatant from 293T cells transfected with pNotS-mA55 also inhibited BrdU incorporation even when rat vascular smooth muscle cells were stimulated with 1, 3 or 10 ng/ml of PDGF and increased BrdU incorporation in a dose-dependent manner, whereas the supernatant from 293T cells

transfected with only pNotS did not affect compared with no supernatant addition (See Fig. 1).

These data revealed that the recombinant mouse A55 protein had the growth inhibitory activity on vascular smooth muscle cells.

#### Experiment 8

##### Preparation of anti mouse A55 polyclonal antibody

Three kinds of peptide fragments of mouse A55 were synthesized by solid phase method:

a    RTNPVYRGPYSNPYSTSYSG    (71-90)    (48-67 of SEQ ID NO. <sup>3</sup><sub>1,2</sub>)  
      GAYYIFQIKSGNEGREFYMR    (376-395) (353-372 of SEQ ID NO. 1)  
      MTRPIKGPRDIQLDLEMITVN    (406-426) (383-403 of SEQ ID NO. 1).

Rabbits were immunized to these peptides as immunogen and the serum were prepared after measurement of the activity. Each anti-mouse A55 antibody was purified by affinity column immobilized each peptide which was used as immunogen from the obtained serum.

The supernatant prepared by the same method described in example 6, was subjected to SDS-PAGE, the separated proteins were transferred to Immobilon-P (PVDF membrane, trade name, Millopore) from the acrylamide gel. After blocking the membranes they were incubated with the anti mouse A55 polyclonal antibody as the first antibody and by developing using ECL kit (Amersham), the recombinant mouse A55 protein was detected.

A 60 k Da band was detected in the supernatant from mA55 transfected Cos1 cells as well as 35S-labeling experiment described in example 7. While no bands were detected in the supernatant from mock-transfected Cos1 cells. These results confirmed that the obtained polyclonal antibodies specifically

recognized the mouse A55 protein.